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Control of Posthavest Rot of Sweeetpotato (*Ipomoea batatas* Lam.) Tuber *in Vitro* and *in Vivo*

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Abstract. A laboratory experiment was conducted at Michael Okpara University of Agriculture Umudike, to determine the inhibition potentials of some indigenous medicinal plants and their essential oils against some plant pathogens associated with sweet potato soft rot *in vitro* and *in vivo*. The essential oils were extracted from five indigenous medicinal plants; *A. indica* (seeds), *Z. officinale* (rhizome), *C. citratus, C. sineensis* (peels) and *O. gratissimum* (leaves) and four species were isolated from diseased tubers, *Erwinia, Flavobacteria, Ralstonia*, and *Bacillus* and later subjected to pathogenicity tests based on their respective ability to cause rot in healthy sweet potato tubers. The experiment was replicated four times using Completely Randomized Design (CRD). *Erwinia, Ralstonia* and *Flavobacteria* were positive as pathogens, while *Bacillus* spp was discarded as a non pathogenic organism on potato tuber. Data obtained showed that in the *in vitro* trials, all the extract and oils assayed inhibited growth of the pathogens in culture to varying degree when compared with that of the control (sterile water). *Z. officinale* oil was however found to be most effective in the inhibition of *Erwinia* pathogen with diameter of 19.33 mm while the control had the least (0.00 mm). In the *in vivo* experiment, *C. citratus* and *A. indica* performed much better than oil extracts of the rest and sterile water ($P \geq 0.05$). It was therefore concluded that there was a variation in the potency of the extract oils as well as in the sensitivity pattern of the test organisms. The potentials of the plant essential oils to serve as possible bio-control and antimicrobial agents for bacterial soft rot of sweet potato were thus demonstrated in this trial.

Keywords: Plant extracts, pathogenicity, **s**ensitivit**y,** i*n vitro, in vivo*: inoculum.

INTRODUCTION

Sweet potato (*Ipomea batatas* Lam.) is an important staple crop in 130 countries worldwide, ranking fourth in production after rice, maize, and wheat ((Ukpabi, 2009; Calvo *et al*., 2010), with Nigeria being the fourth biggest producer in Sub Saharan Africa (FAO, 2008). Potato is a carbohydrate rich food providing a good source of dietary energy and some micro nutrients to consumers. In comparison with other roots and tubers, the protein content of potato is very high (FAO, 2008).

Sweet potato tubers suffer from post harvest losses as a result of some physical, pathological factors and physiological or a combination of all three factors (Booth, 1974). The principal factor responsible for losses during storage of *I. batatas* has been reported to be as a result of infection by microorganisms resulting in tuber decay mainly bacteria causing rots in potato have been reported to produce a wide range of hydrolytic enzymes such as cellulases, pectinases, xylanases, and proteases. These enzymes are also responsible for tissue maceration and cell death (Amadioha and Adisa, 1993; Olivieri *et al*., 2004).

Bacterial soft rot is one of the most common potato diseases in the tropics and induces quick and heavy spoilage losses. Its causal agent, *Erwinia carotovora* sub sp. *carotovora* (Van Hall) Dye, is one of the most important and widespread bacterial disease of a variety of plants either in the field or in storage (Hajhamed *et al*., 2007; Harrison and Nielson, 1990). It causes substantial losses in transit and storage, particularly in the warm regions where temperatures are high and there are no facilities available for cold storage (Cromarty and Easton, 1973). Approximately 22% of potatoes are lost per year due to viral, bacterial, fungal, and pests attack to potato tuber and potato plant, incurring an annual loss of over 65 million tones and bacterial soft rot alone accounts for 30–50% of this huge loss (Czajkowski *et al*., 2011).

Several methods of the disease control such as hot water treatment (Shirsat *et al*., 1991) and air-drying of tubers (Bartz and Kelman, 1985b) have been tried with varying scale of success. The use of products of plant origin such as neem

(*Azadirachtin indica*) is much safer but these products are applied mostly in insect pest management than control of microorganisms (Stoll, 1998). However, extracts of ginger rhizomes (*Zingiber officinale*), garlic bulb (*Allium sativum* L.) and aloe vera (*Aloe barbadensis* M.) have been used in controlling fungal pathogens (Amadioha, 1999; Obagwu *et al*., 1997; Ahmed and Beg, 2001). Some bactericidal and fungicidal properties of these products had been reported (Emechebe and Alabi, 1997; Stoll, 1998).

The lack of appropriate storage facilities in Nigeria has led to high losses of potato tubers in transit and storage thus this search for a more effective and cheaper method of controlling the disease (bacterial soft rot caused by *Erwinia carotovora*) in storage becomes very essential. Therefore this work is aimed at determining the effect of some plant extract oils on the soft rot pathogens of sweet potato both at *in vitro* and *in vivo*.

MATERIALS AND METHODS

The plant materials used in these trials were: *A. indica* (seeds), *Z. officinale* (rhizomes), *C. citrates* (leaves), *C. sineensis* (peels) and *O. gratissimum* (leaves) were all sourced locally within the University community while the sweet potato tubers were obtained from the Sweet Potato Research Programme of National Root Crops Research Institute (NRCRI) Umudike, Abia State, Nigeria. Laboratory facilities used were those of Crop and Soil Sciences Michael Okpara University, Umudike and the Central Laboratory Unit of NRCRI.

The preferred medium, Bacho nutrient agar (NA) was prepared in accordance with the manufacturer's recommendations in which 28g of the powder was dispersed in 1liter distilled water in a flask, mixed and heated in water bath until the Agar melted to form a homogenous mixture. Its pH was measured and adjusted to standard of 7.2±0.2. The prepared medium was sterilized in an autoclave at 121^{o}C for 15minutes. The sterile medium was allowed to cool down to 45^{o}C before it was aseptically poured in 15ml aliquots into sterile Petri dishes where it was finally

allowed to cool and solidified at room temperature before being used for bacteria culture.

Preparation and Extraction of Oils from the Plant Extracts

Each of the plant materials used for the experiment was washed thoroughly in running water, drained and air dried at 30°C room temperature until brittle enough to grind. They were processed first into powdered form to increase surface area and enhance oil extraction.

Oil extraction from the plants was obtained as described by Benjilali *et al*. (1984) and modified by Amadioha (2002). In this method, weighed and processed (200g) sample of each material was soaked in excess acetone. The mixture was shake thoroughly after allowing it to stand for 24 hours and then filtered using cheese cloth; the filtrate obtained was transferred to oil extraction flask and connected to a soxhlet apparatus. The solvent (acetone) was systematically removed each time the soxhlet reflex filled up. This was continued until no more solvent was left in the flask. The oil extracted was then transferred to a screw capped sample bottle and stored at 32°C and was used within 72hr of extraction.

Bacteria Isolation

The method described by the International Commission on Microbiological Specification of Food (ICMSF, 1998) was employed. Each diseased sweet potato tuber was first surface sterilized by mopping the entire surface with cotton wool moistened with 1% sodium hypochlorite solution (bleach). The tuber was then cut open with flamed knife to reveal boundary area between diseased and healthy portion. Bits were cut out and teased out in 10ml of sterile water in a beaker. Subsequently, a loopful of the bacterial inoculum was collected from the suspension and aseptically inoculated evenly and streaked onto sterile nutrient agar plates. The inoculated plates were kept in the incubator at 37°C for 48hr and observed daily for colony growth. On establishment of growth, the plates were examined closely for distinct colonies from which single formed colonies were collected and

transferred aseptically onto sterile nutrient agar plates. After incubation, the resulting sub-cultured plates were examined for uniformity to ensure purity and consistency. The pure cultures obtained were used for identification and subsequent tests.

Characterization and identification of Isolated Pathogens

Identification of the bacteria isolates was based on examination of the different characteristics with reference to existing taxa in a standard manual and the Bergey's Manual of Determinative Bacteriology (Safrinet, 2000; Buchaman and Gibbons, 2004). Four levels of characterization were outlined as follows:

Colony features; by close examination of the culture colonies and a record of its characteristics in terms of extent of growth, colour, nature of colony edge, elevation, etc. Microscopic examination; by observing each isolate with a binocular microscope and taking a record of its reaction to general dye (Gram stain) as well as specific dyes which indicated the presence or absence of features like spores, flagella, etc. The shape and arrangement of the cells were also taken into consideration. Final identification tests were conducted by some biochemcal reaction tests; carried out to confirm the ability of the enzymes like the catalase, indicated by production of bubbles showing either a positive (+ve) or a negative (-ve) reaction. Oxidase-reduction test, nitrate and indole reaction test as well as H_2S production tests were all conducted. In addition to the above biochemical tests; sugar utilization tests involving determination of the ability of the isolates to utilize different sugar sources such as sucrose, glucose, maltose, lactose and xylose leading to acid and/ or gas production were equally determined.

Pathogenicity Tests: The ability of each of the bacterial isolate to cause soft rot in healthy potato tubers was carried out as a confirmatory test of pathogen identity. The tubers were first surface sterilized by swapping the entire surface with cotton wool moistened with 1% sodium hypochlorite solution. Then the method of Okigbo and Ikediugwu (2000) was employed, in which holes were made in the potato tubers with the aid of a flamed 5mm cork borer, and aseptically inoculated with 0.5ml of

48hr old culture of the bacterial isolate. The hole was covered by replacing the removed fleshy core and then sealing up the site with sterile petroleum jelly. The inoculated tubers were labeled accordingly and kept at room temperature. They were observed for symptoms of rot like colour change, softening, characteristic foul odour, etc. A control experiment was also set up by opening and closing the core in a tuber without introducing any organism in it except with 0.5ml sterile water.

Two weeks after inoculation, the tubers were carefully examined by cutting open transversely along the line of inoculation to reveal the extent of rot inside. Bacteria isolates which caused clearly visible rot were considered pathogenic when viewed against the same situation in the control. Those with records of pathogenicity were used as test organisms for treatment with the plant extracts.

Sensitivity Tests: The test on sensitivity of the established pathogens was done at two levels namely; *in vitro* and *in vivo*:

***In Vitro* Test:** Antimicrobial sensitivity test was carried out using the Disc Diffusion Technique according to Cheesbrough (2000). Each pathogenic isolate from a 48hr culture was inoculated by the spread plate technique. Circular paper discs (5mm in diameter) were punctured out from a Whatman No.1 filter paper; the discs were sterilized using 70% (absolute) ethanol and oven dried. Selected discs were added to the oil extracts separately and allowed to absorb the extract. The oil bearing discs was placed three on the inoculated plate with the aid of flamed pair of forceps, each at about 4mm spacing in between the disc, pressed gently and allowed to stand for 5 minutes before the plates were incubated. After 48hr the incubated plates were observed for growth and inhibition, this was done for each pathogenic isolate to observe the presence of a clear zone around the oil extract discs indicating inhibition of the organism or otherwise and the plant pathogen was to be considered sensitive to the extract oil, because of its ability to inhibit the growth of the pathogen. A check experiment was also made separately by following the same arrangement described above but with standard commercial antibiotic multidisc (streptomycin) and records taken. Another plate inoculated with the pathogenic organism was placed with the discs dipped in sterile water as treatment instead of

extract oil. Record of extent pathogen inhibition was obtained by measuring the diameter of inhibition zone in each case. The percentage (%) growth inhibition was determined using the formula modified by Amadioha (2003; 2004) as:

$$\% \text{ inhibition} = \frac{dc - dt}{dc} \times \frac{100}{1}$$

dc = Colony diameter of control.

dt = Colony diameter of treated tubers.

***In Vivo* Test:** Unlike in the *in vitro* test, the sensitivity of each pathogen was tested on healthy *I. batatas* tubers. Core openings were made with flamed cork borer, inocula dipped inside the extract before being placed inside the hole. Then, the removed core was replaced and point of inoculation sealed with sterile petroleum gel. A control was set up in each case, in which the pathogen was inoculated unto separate potato tuber but without the extracts. All the tubers were allowed to stay at room temperature for 8days, after which the potato tubers were cut open along the point of inoculation. The extent of rot in each case was measured (as length) with transparent meter rule also the extent of growth inhibition of the pathogen was measured in relation to the level of rot caused in the control (without extracts). The percentage (%) growth inhibition was obtained as in the *in vitro* tests.

Experimental Design and Data Analysis

All tests were laid in Completely Randomized Design (CRD) with four replicates. Data were subjected to Analysis of Variance (ANOVA) using 2009 SAS model and effects was declared significant or non- significant at ($P \leq 0.05$) level of probability for those parameters where the ANOVA was significant using LSD (least significant difference) at 5% level of probability ($P \leq 0.05$) according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Table I shows the summary of the characteristic biochemical properties of the bacteria isolated from diseased sweet potato tuber while Table 2 shows the disease

incidence of bacteria isolates in infected sweet potato tubers and the pathogenicity profile of the isolates on healthy tubers. Table 3 is the summary of *in vitro* tests and the inhibition of the plant extracts on the different bacteria pathogens. Results of the *in vivo* sensitivity (inhibition) and percentage growth inhibition of the bacteria isolates are summarized in Table 4.

Isolation of Pathogen

Four different bacteria (species) colonies were isolated from the different diseased sweet potato tubers showing typical soft rot symptoms. The bacteria species isolated were *Erwinia*, *Ralstonia, Bacillus* and *Flavobacteria.* Out of these four bacteria species tested, *Erwinia* and *Ralstonia* were more abundant and consistent with 80% occurrence each while *Flavobacteria* and *Bacillu*s appeared 60% and 40% respectively.

This experiment indicates that the high relative %incidence of *Erwinia* on sweet potato tubers was attributed to its ubiquitous nature in the soil. It was however observed that *Erwinia* and *Ralstonia* present in the test samples have been implicated in potato bacterial soft rot disease (Agrios, 2006).

Identification and Characterization of Bacteria Isolates

Table1 represents the identification and preliminary confirmatory tests for the bacteria isolates based on the description of Bergey's Manual of Determinative Bacteriology (Buchaman and Gibbons, 2004).

Table 1: Characteristics of Bacteria Pathogen Isolated from Diseased *Ipomea Batatas*

Tests	*Erwinia*	*Ralstonia*	*Flavobacteria*
Colony features:	Round creamy white, slightly raised and glistening.	Circular green pigmented colonies slimy and consistent.	Circular colonies with smooth surface and yellow tints.
Microscopic Examination:			
Gram stain	-ve	-ve	-ve
Spore	-ve	-ve	-ve
Flagella	-ve	-ve	-ve
Motility	-ve	-ve	-ve
Catalas	+ve	+ve	+ve
Oxidas	+ve	+ve	+ve
Nitrate	+ve	+ve	+ve
Indole	-ve	-ve	-ve
CabohydrateUtilization:			
Glucose	+ve	+ve	-ve
Lactose	+ve	-ve	-ve
Sucrose	-ve	-ve	-ve
Maltose	+ve	-ve	-ve
Xylose	+ve	+ve	-ve

+ve = Positive reaction; -ve = Negative reaction.

Characterization was done in four levels: colony features, microscopic examination (Gram stain, spore, flagella and motility) biochemical reaction tests (catalase, oxidase, nitrate and indole production) and carbohydrate utilization.

In characterization and identification, *Erwinia carotovora* was found to be rod shaped creamy white (Gupta and Thind, 2006), slightly raised and glistening colonies, a Gram negative, rod after staining. Smear culture with a drop of hydrogen peroxide (H_2O_2) produced bubbles indicating positive for catalase tests. It reduced nitrate, utilized glucose and lactose similar to what was reported by Bradbury (1986).

Determination of Pathogenicity Test

A confirmatory test was however based on the results of the pathogenicity tests conducted in which each of the isolates inoculated into healthy sweet potato tubers were observed for soft rot symptoms within 14days in accordance with Koch's postulate. Results indicated that the bacteria isolates under investigation were able to induce soft rot, although they varied in severity of rot initiation (Table 2).

Table 2: % Incidence/occurrence and Pathogenicity of Bacteria Isolates

Isolates	% Occurrence	Pathogenicity
Erwinia	80	+ve
Ralstonia	80	+ve
Flavobacteria	60	+ve
Bacillus	40	-ve

The tests further showed that *Erwinia* spp, *Ralstonia* spp and *Flavobacteria* spp were all pathogenic while *Bacillus* spp was possibly a secondary micro flora or saprophyte that manifested as contaminants and for this reason was discarded. Though (Olivieri *et al*. (2004) and Mahmoud *et al*. (2008) in their separate works reported that *Erwinia* spp and *Bacillus* spp could be found in association with bacterial soft rot in potato.

Pathogenicity test conducted revealed that *Erwinia carotovora* induced rot at temperature between 30^{o}C and 32^{o}C which is the normal tropical room temperature (Bradbury, 1986). Soft rot symptoms began after 7days with small water soaked lesion. The inoculated area became soft and mushy (watery) while the surface became discolored and depressed, tissues within the region were slimy (Walker, 1998). A characteristics black border separated the diseased area from the healthy tissue (Elphinestone and John, 2010).

Effect of Different Plant Extracts on cultures (*In Vitro*)

The various oil extracts exhibited varying degrees of inhibition against the bacteria pathogens using the paper disc method *in vitro*. Results of the tests are

shown in Table 3. It was observed that all oil plant extracts performed better than sterile water (control), Z. *officinale* oil had the highest inhibition zone (19.33mm) against *Erwinia* and was as effective as the standard antibiotics (28.68mm) followed by *A. indica* seed oil (16.33mm). However, *O. grtatissimum* extract was the least (P≤0.05) in potency (after the control) against the pathogen with inhibition zone diameter of 11.00mm. *Ralstonia* and *Flavobacteria* were found to be more sensitive to *A. indica* (21.68mm and 12.93mm) next to the antibiotics with inhibition zone of 25.68mm and 20.68mm (P≤0.05) respectively.

Table 3: Mean Inhibition Diameter of Different Plant Extracts on Plate (*In Vitro*)

	Inhibition Diameter (Mm)		
Plant Extracts	*Ewrinia*	*Ralstonia*	*Flavobacteria*
A. Indica	16.33	21.68	12.93
C. Citratus	12.58	12.25	8.83
Z. officinale	19.33	14.33	9.25
C. sineensis	11.68	13.75	9.18
O. gratissimum	11.00	11.83	9.08
Streptomycin	28.68	25.68	20.68
Water (control)	0.00	0.00	0.00
LSD (P ≤ 0.05)	1.076	3.435	6.677

There were variations in the inhibitory reaction of the plant extracts as well as streptomycin sulphate relative to control experiment at *in vitro* level. These inhibitions were found to be significantly different at P ≤ 0.05 statistically.

Based on the result, it was observed that the *in vitro* inhibition test revealed *Z. officinale* extract as the most superior in inhibiting the growth of *Erwinia* and *A.indica* for other pathogens, probably because of the diffusible essential oil content; similar conclusion was made by earlier workers against some pests (Benjilal *et al.*,1984; Jones and Jones, 1984) while *O.gratissimum* extract was the least.

Effect of Different Plant Extracts on Sweet Potato Tubers (*In Vivo*)

From the *in vivo* experiment, the activity of the plant extracts showed that the test pathogens reacted to the extracts to different levels as shown by mean inhibition diameter of rot caused on potato tubers and % growth inhibition of the plant extracts which are recorded as shown in Tables 4 and 5. The extracts showed variation in the reduction level of rot caused by the pathogens. The extracts of *C. citratus* (60.83%) was more active and the value differed significantly ($p \leq 0.05$) from other plant extracts against caused rot while the extracts of *Z. officinale* was the least effective against the pathogen with an inhibition of 35.83% for *Erwinia*. The other extracts have varying level of inhibition between these two levels (35.83% - 60.83%). Also, *C. citratus* extract reduced rot growth of *Ralstonia* and *Flavobacteria* by 57.14% and 57.29% respectively. The least effective extract against *Ralstonia* and *Flavoabateria* is *C.sineensis* rating 43.96% and 41.67% respectively.

Table 4: Mean Inhibition Diameter of Potato Soft Rot Caused by Bacterial Pathogens

	Inhibition Diameter (mm)		
Plant Extracts	***Ewrinia***	***Ralstonia***	***Flavobacteria***
A. indica	14.00	10.75	11.50
C. citratus	11.75	9.75	10.25
Z. officinale	14.75	10.75	12.25
C. sineensis	17.75	12.75	14.00
O. gratissimum	17.75	9.75	10.25
Streptomycin	7.00	5.00	5.50
Water (control)	30.00	22.75	24.00
LSD (P=0.05)	3.215	1.541	2.386

Table 5: Percentage (%) Growth Inhibition of Different Plant Extracts (*In Vivo*)

	% Inhibition Diameter (mm)		
Plant Extracts	*Ewrinia*	*Ralstonia*	*Flavobacteria*
indica	53.33	52.75	52.08
C. citratus	60.83	57.14	57.29
Z. officinale	35.83	52.75	44.79
C. sineensis	40.83	43.96	41.67
O. gratissimum	50.83	51.65	48.96
Streptomycin	76.67	78.02	77.08
Water (control)	30.00	22.75	24.00
LSD (P=0.05)	3.215	1.541	2.386

There was variation in the rot caused by the *Erwnia carotovora* as a result of the active citral, azadirachtin, zingiberene, eugenol and limonene in the oil of the plant extracts. Citral of *C. citratus* was observed to performed best and is significantly different ($p \leq 0.05$) from that of *A.indica* and the least effective was Zingiberene of *Z. officinale* also reported by Jones and Jones,1985; Fahy and Hayward, 1993; Kumar and Parmar, 1996; Stoll,2000).

CONCLUSION AND RECOMMENDATION

It was discovered that there was significant difference in the potency of the extracts against the pathogens at the *in vitro* level and *in vivo* level compared to their control. The variation in the activity of the extracts at both levels of tests was attributed to possible effect of the organic nature of the *in vivo* test on the extracts. Generally, the results show that the botanicals possess antibacterial activity and have the potentials for exploitation and utilization as biocontrol agents in the fight against bacterial soft rot of sweet potato.

In order to prevent degradation of biodiversity and the adverse effect of the use of synthetic chemical in control of bacterial soft rot. It is therefore recommended based on the research project that plant extracts oil from *A indica* and *C citratus* are capable of inhibiting the growth of bacteria pathogens that cause soft rot.

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